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RNA Interference Targets Arbovirus Replication in *Culicoides* Cells

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Arboviruses are transmitted to vertebrate hosts by biting arthropod vectors such as mosquitoes, ticks, and midges. These viruses replicate in both arthropods and vertebrates and are thus exposed to different antiviral responses in these organisms. RNA interference (RNAi) is a sequence-specific RNA degradation mechanism that has been shown to play a major role in the antiviral response against arboviruses in mosquitoes. *Culicoides* midges are important vectors of arboviruses, known to transmit pathogens of humans and livestock such as bluetongue virus (BTV) (*Reoviridae*), Oropouche virus (*Bunyaviridae*), and likely the recently discovered Schmallenberg virus (*Bunyaviridae*). In this study, we investigated whether *Culicoides* cells possess an antiviral RNAi response and whether this is effective against arboviruses, including those with double-stranded RNA (dsRNA) genomes, such as BTV. Using reporter gene-based assays, we established the presence of a functional RNAi response in *Culicoides sonorensis*-derived KC cells which is effective in inhibiting BTV infection. Sequencing of small RNAs from KC and *Aedes aegypti*-derived Aag2 cells infected with BTV or the unrelated Schmallenberg virus resulted in the production of virus-derived small interfering RNAs (viRNAs) of 21 nucleotides, similar to the viRNAs produced during arbovirus infections of mosquitoes. In addition, viRNA profiles strongly suggest that the BTV dsRNA genome is accessible to a Dicer-type nuclease. Thus, we show for the first time that midge cells target arbovirus replication by mounting an antiviral RNAi response mainly resembling that of other insect vectors of arboviruses.

Biting arthropods such as mosquitoes, ticks, and midges can transmit a variety of viruses (arboviruses) belonging to the *Flaviviridae*, *Bunyaviridae*, *Togaviridae*, and *Reoviridae* families. Arboviruses actively replicate in both their arthropod vector and vertebrate host. At present, mosquito-borne viruses are probably the best-studied arboviruses. Among these are viruses of particular relevance to public health, including members of the *Flaviviridae* family, such as dengue virus (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV), or alphaviruses of the *Togaviridae* family, such as chikungunya virus (CHIKV) (1).

Midge-borne viruses also impact on public health. Oropouche virus (OROV) infection can result in Oropouche fever, one of the most important arboviral diseases in America (mainly in the Amazon region, Panama, and Caribbean) (2, 3). *Culicoides* are biting haematophagous midges belonging to the family Ceratopogonidae. Importantly, 96% of the >1,400 identified species attack mammals, including humans. *Culicoides* are well-known vectors of protozoans, filarial worms, and viruses (3), and more than 50 viruses belonging to the *Bunyaviridae*, *Reoviridae*, and *Rhabdoviridae* families have been isolated from different *Culicoides* species. While some of these may be accidental infections, around 45% of isolated viruses are specific to *Culicoides* species, including those known to cause infections of livestock all over the world, such as African horse sickness virus (AHSV), bluetongue virus (BTV) (*Reoviridae*) (3), and the recently discovered Schmallenberg virus (SBV) (*Bunyaviridae*) (4).

As arboviruses require vectors for successful transmission between vertebrate hosts, there is evolutionary pressure on keeping the right balance between virus replication and vector survival. Recent research on mosquito-arbovirus interactions indicates that innate immune responses such as RNA interference (RNAi) are key factors in restricting arbovirus replication (5–12), as detailed in recent reviews (13, 14). Similar research on midge-transmitted arboviruses has not been carried out despite the fact that important arboviruses are transmitted by these vectors.

RNAi has been shown to be an important and possibly the major antiviral response in mosquitoes (13, 14). RNAi consists of different pathways that perform sequence-specific targeting of RNA. However, the exogenous small interfering RNA (siRNA) pathway is of particular interest given its antiviral function, as demonstrated in different organisms, including drosophila and mosquitoes (13–15). The mosquito exogenous RNAi pathway is induced by virus-derived long double-stranded RNA (dsRNA) either derived from replication intermediates or secondary structures that are targeted by the RNase III enzyme Dicer-2 (Dcr-2) and cut into virus-derived small interfering RNAs (viRNAs) of mainly 21 nucleotides (nt) in length, as is assumed through comparisons to *Drosophila melanogaster* (5, 7, 11, 12, 16–20). These viRNAs are taken up by the RNA-induced silencing complex (RISC), harboring an argonaute protein (Ago-2) as the catalytic compound. viRNAs are then unwound, and one strand is kept in the RISC to be used as a guide to find complementary viral RNA sequences. After base pairing, the catalytic domain of Ago-2 cleaves the target (viral) RNA, at least in the drosophila model, which silences viral infections (13, 14, 21–23). The exogenous siRNA pathway can

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also be artificially induced by the addition/transfection of long dsRNA or siRNA molecules, resulting in sequence-specific silencing. Key proteins of the RNA silencing pathways, such as Dcr-2 and Ago-2, have been shown to be conserved in drosophila and mosquitoes, and the effector mechanisms are likely to be similar. Other Dicer and Ago proteins are involved in a variety of small RNA silencing pathways, such as the microRNA pathway (13, 14, 23). A number of RNAi-competent mosquito cell lines, such as Aag2 (derived from *Aedes aegypti*) and U4.4 (derived from *Aedes albopictus*), as well as Dcr-2-deficient cell lines (C6/36 and C7-10, both derived from *Aedes albopictus*), have proven to be highly useful in studying mosquito RNAi responses (6, 10–12, 19, 24, 25). However, nothing is known about the presence and function of RNAi pathways, specifically the antiviral exogenous siRNA pathway, in midges and their derived cell lines.

BTV is one of the best-studied midge-borne viruses. It has been shown to replicate both in its arthropod vector and mammalian host (26, 27). BTV infection leads to persistent infection in infected adult *Culicoides* or midge-derived cell culture (28–30). This is in contrast to infected mammalian cells, which show strong cytopathic effects (30). Given the absence of studies on *Culicoides* RNAi pathways and antiviral mechanisms, nothing is known about the interactions of BTV with vector immune responses. Many *Culicoides* species have been identified as BTV vectors around the world, including *Culicoides imicola* in Africa (31) and Southern Europe (32), *Culicoides obsoletus* and *Culicoides pulicaris* in Central and Northern Europe (33, 34), and *Culicoides variipennis* and *Culicoides sonorensis* in America (35, 36). The BTV genome consists of 10 segments of dsRNA molecules (each comprising a coding and noncoding strand) that are packaged within a nonenveloped triple-layered icosahedral protein capsid (37–39) and direct the expression of 7 structural proteins (VP1 to VP7) and 4 distinct nonstructural proteins (NS1, NS2, NS3/NS3a, and NS4) (39–41). In contrast to the single-stranded RNA arboviruses with positive-sense (alphaviruses, flaviviruses) or negative-sense (bunyaviruses) RNA genomes that have been studied in mosquitoes or mosquito cell culture systems, the dsRNA nature of the BTV genome adds a layer of complexity for the antiviral RNAi response in insects. During the reovirus replication cycle, second-strand RNA synthesis is believed to occur only after assembly and consequently within the newly formed viral particles. As such, viral dsRNA is not necessarily accessible to the RNAi machinery. In addition to BTV, we are also investigating the RNAi response against SBV, an unrelated negative-strand RNA arbovirus. SBV is a recently emerged virus that affects ruminants causing mild disease (reduced milk production, pyrexia, and diarrhea) in adults and congenital malformations in stillborns or newborns (42, 43). SBV belongs to the genus *Orthobunyavirus* within the *Bunyaviridae* family and possesses a three-segmented negative-sense RNA genome. The large (L) segment encodes the RNA-dependent-RNA polymerase L, the medium (M) segment encodes a polyprotein that is cleaved into two glycoproteins (Gn and Gc) and a nonstructural protein (NSm), while the small (S) segment encodes the nucleoprotein (N) and a second nonstructural protein (NSs). SBV is believed to be transmitted by *Culicoides* species to susceptible mammalian hosts (4). Again, little is known about control of bunyavirus replication by RNAi responses of arthropod vectors. Recently, LaCrosse virus (LACV)-infected drosophila cells were shown to produce virus-specific 21-nt viRNAs mapping to all three viral segments, similar to what has been observed for other (mainly positive-strand RNA) arboviruses (6). In addition, LACV-infected *Aedes albopictus* C6/36 cells, known to be deficient in Dcr-2 activity, were shown to produce virus-specific small RNAs of different sizes (6, 44).

In this study, (i) we investigated the presence of a functional exogenous siRNA pathway in the *C. sonorensis*-derived KC cell line and (ii) we assessed whether this antiviral response targets midge-borne arboviruses with either dsRNA (BTV) or negative-strand RNA (SBV) genomes. We identified an exogenous siRNA pathway in KC cells that could be induced by dsRNA or viral infection and is effective against two arboviruses with highly different genome structures. A comparative analysis with BTV- or SBV-infected *Aedes aegypti* Aag2 cells suggests that the midge antiviral RNAi response against these viruses resembles mainly that of mosquitoes and points to conservation of key elements controlling arbovirus replication by RNAi in insect vectors.

MATERIALS AND METHODS

Cells. BSR cells, a clone of BHK-21 (kindly provided by Karl K. Conzelmann), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). BHK-21 cells were grown in Glasgow minimal essential medium (GMEM) supplemented with 10% newborn calf serum and 10% tryptose phosphate broth. CPT-Tert cells (45), immortalized sheep choroid plexus cells (kindly provided by D. Griffiths), were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS. Mammalian cell lines were cultured at 37°C in a 5% CO_2 humidified atmosphere. KC cells, obtained from *C. sonorensis* larvae, were grown in Schneider's insect medium supplemented with 10% FBS (46), *Aedes aegypti*-derived Aag2 mosquito cells were grown in L-15 medium supplemented with 10% FBS and 10% tryptose phosphate broth. Insect cells were maintained at 28°C.

Viruses and plasmids. BTV-1 was rescued by reverse genetics as previously described (41) and derived from the reference strain of BTV-1 originally isolated at the ARC-Onderstepoort Veterinary Institute. Virus stocks were prepared by infecting BSR cells at a low multiplicity of infection (MOI; 0.001) and harvesting the supernatant at 72 h postinfection. The virus suspension was centrifuged at 500 × *g* for 5 min. SBV (kindly provided by M. Beer) was initially isolated from blood of an infected cow and passaged once in KC cells and 6 times in BHK-21 cells. The virus was plaque purified, and stocks were produced in BHK-21 cells by infecting cells at a low MOI (0.01) and harvesting the supernatant at 120 h postinfection, followed by 20 min of centrifugation at 3,500 rpm. Virus titers of SBV and BTV-1 were established by standard plaque assays using CPT-Tert cells (47).

Expression vectors for invertebrate cells, pIZ-Fluc and pAcIE1-Rluc, expressing firefly (*FFluc*) and *Renilla* (*RLuc*) luciferases, respectively, have been previously described (48), and the fluorescein-labeled plasmid DNA was commercially obtained (Mirus).

Luciferase assays. Luciferase activities were determined using a dual-luciferase assay kit (Promega) on a GloMax-Multi+ microplate multimode reader following cell lysis in passive lysis buffer.

dsRNA production. dsRNA for the RNA silencing experiments was produced with the RNAi Megascript kit using gel-purified PCR products of a specific sequence (Table 1) flanked by T7 promoter sequences. Fluorescein-labeled dsRNA was produced by T7 RNA polymerase transcription (Invitrogen) on an enhanced green fluorescent protein (eGFP)-derived PCR product (using pC1-eGFP from Clontech as the template) with the fluorescein-labeled rNTP mix (Roche) by following the manufacturer's protocol. The DNA template and single-stranded RNA were removed by DNase1 and RNase A treatment (Ambion). dsRNA was then ethanol precipitated, dried, and resuspended in water.

TABLE 1 List of primer sequences used

Gene	Upstream/downstream primer sequence (5′→3′)[a]
BTV-1 NS1	*GTA ATA CGA CTC ACT ATA GGG* TCGGTGGG AATGGCTTAT
	GTA ATA CGA CTC ACT ATA GGG CTTTTCTG CATAGCATAGGGTG
eGFP, 400 nt	*GTA ATA CGA CTC ACT ATA GGG* GGCGTGC AGTGCTTCAGCCGC/
	GTA ATA CGA CTC ACT ATA GGG GTG GTTGTCGGGCAGCAGCAC
Firefly luciferase	*GTA ATA CGA CTC ACT ATA GGG* ATGGAAGC AGCCAAAAAC
	GTA ATA CGA CTC ACT ATA GGG TTACACG GCGATCTTTCC

[a] The T7 promoter region is indicated in italics.

Transfection and infection. In order to determine the transfection efficiency, 5×10^5 *C. sonorensis*-derived KC cells were seeded per well in 24-well plates with a glass bottom prior to transfection. DNA or dsRNA was incubated in the presence of a variety of transfection reagents (Fugene, Genejammer, and Lipofectamine 2000) and added to cells according to the manufacturer's protocol. In the case of Fugene and Genjammer, a ratio of 1 to 3 (micrograms of nucleic acid to transfection reagent) was used. At 24 h posttransfection, fluorescence in cells was analyzed using a Zeiss laser scanning microscopy (LSM) Meta microscope.

For reporter RNAi assays, 5×10^4 KC cells were seeded per well in 96-well plates and transfected with 250 ng pIZ-Fluc and 50 ng pAcIE1-Rluc using Fugene. Different concentrations of dsRNA, control (eGFP-specific) or targeting *FFluc*, were either cotransfected (5 ng, 1 ng, or 0.5 ng) in 50 μl of Schneider's medium, followed by the addition of 50 μl Schneider's medium with 30% fetal calf serum (FCS) at 2 h posttransfection, or added (300 ng or 100 ng) to cells in 100 μl Schneider's medium with 15% FCS at 24 h posttransfection. Luciferase activity was determined at 48 h posttransfection.

Assays to test dsRNA-induced antiviral activity were performed by seeding 1×10^6 KC cells per well in 24-well plates with glass bottoms. After 24 h, either 100 ng dsRNA, control (eGFP-specific) or specific for BTV NS1, was transfected using Lipofectamine 2000 or 500 ng dsRNA was added to the medium in the absence of a transfection reagent. KC cells were infected at an MOI of 0.2 with BTV-1 24 h posttransfection. At 2 h postinfection, the inocula were removed and cells were washed once with PBS. Supernatant was collected from cultured cells 24 h postinfection and centrifuged at $500 \times g$ for 5 min. Viral titers were subsequently determined by endpoint dilution analysis on BSR cells and expressed as $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$)/ml, calculated using the method of Reed and Muench (49). Cells were then fixed in 5% formaldehyde for 30 min at 24 h or 48 h postinfection (as indicated) and subsequently used for immunofluorescence assays or lysed for Western blot analysis.

***In vitro* growth kinetics of SBV.** The *in vitro* growth kinetics of SBV were determined in KC and Aag2 cells following infection with an MOI of 10 for 1 h. Samples were collected at 0, 8, 24, and 48 h postinfection, and virus titer was determined by plaque assays in CPT-Tert cells. Each experiment was performed in triplicate and repeated twice.

Western blotting. Protein expression in BTV-1-infected KC cells was assessed by SDS-PAGE and Western blotting using polyclonal rabbit antiserum raised against NS1 (41) and a rabbit polyclonal anti-actin antibody as the control (Sigma; A5060).

Detection of proteins by immunofluorescence. Formaldehyde-fixed cells were permeabilized by incubation in 0.3% Triton-PBS for 30 min, followed by washing with PBS, a further incubation in 0.1% SDS-PBS for 10 min, and incubation in PBS. Cells were preincubated in CAS-Block for 1 h at room temperature, followed by an incubation with CAS-Block-diluted polyclonal rabbit antiserum raised against BTV-1 NS1 (1:2,000) (41) or SBV N (50) (1:500) for 90 min at 37°C. Cells were then washed three times for 5 min with PBS. Following this, an anti-rabbit antibody conjugated with Alexa 488 diluted in CAS-Block (1:3,000) was added and incubated for 1 h at 37°C. Following a further washing step with PBS, cells were dried and covered with 4′,6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vectashield; hard set), and fluorescence was detected using a Zeiss LSM meta microscope.

Small RNA isolation and deep sequencing analysis. Sequencing of small RNAs was performed by using the Illumina Solexa platform; KC (8×10^6/well) and Aag2 (2.6×10^6/well) cells in 6-well plates were infected by BTV-1 at an MOI of 0.2 or SBV at an MOI of 10. At 24 h postinfection (KC cells) or 48 h postinfection (Aag2 cells), total RNA was isolated using 1 ml TRIzol (Invitrogen) per well. Glycogen was added prior to isopropanol addition to enhance recovery of small RNA from samples. Total RNA was then loaded on a 15% denaturing urea acrylamide gel, and RNA molecules of 18 to 30 nt in size were purified from the gel, linked to adapters, reverse transcribed, and sequenced by ARK-Genomics (The Roslin Institute, University of Edinburgh) on the Illumina Solexa platform (HiSeq 2000). Illumina adapters and sequencing primers were removed using cutadapt (51), and the trimmed sequences were aligned to the reference genome using Novoalign. Graphs and reports were produced in R (52) using the viRome package (http://www.ark-genomics.org/services-bioinformatics/virome). The complement-distance plots were calculated as follows: the distance between the 5′ end of reads of 24 to 30 bp that map on complementary strands was counted, and the sum of counts was plotted against the distance. For the sequence logos, counts of each base at each position were used to create a position-weight matrix, and the subsequent sequence logo was plotted using the seqLogo (53) package from Bioconductor (54). Small RNAs were mapped to SBV (GenBank accession numbers JX853179 to JX853181) or BTV-1 (GenBank accession numbers JX680457 to JX680466).

Nucleotide sequence accession number. Small RNA sequences (of data shown and repeats) were submitted to the European Nucleotide Archive (accession number ERP001936).

RESULTS

An active RNAi pathway is present in the KC midge cell line. An RNAi response can be induced in arthropod cells by sequence-specific dsRNA either by transfection, or in case of drosophila cells, by addition to the cell culture medium (55). We designed a luciferase-based reporter assay in order to investigate if *C. sonorensis*-derived KC cells can induce a dsRNA-mediated RNAi response. As little is known about the efficiency and/or toxicity of transfection reagents in KC cells, a pilot experiment was carried out with either fluorescently labeled dsRNA molecules or plasmid DNA and by using different transfection reagents. At 24 h post-seeding, KC cells were incubated with fluorescein-labeled dsRNA or fluorescein-labeled plasmid DNA in the absence or presence of different transfection reagents (Fugene HD, GeneJammer, or Lipofectamine 2000) by following the manufacturer's protocol. At 24 h postincubation, we estimated the number of transfected cells (green cells) by fluorescence microscopy, with at least 400 cells counted for each condition (data not shown). No obvious toxicity was detected 24 h posttransfection (data not shown). Use of Fugene and GeneJammer resulted in similar numbers of transfected cells (between 14% and 23%). Lipofectamine 2000-mediated transfection resulted in the highest number of transfected cells for dsRNA (approximately 40%), similarly to what we obtained in cells incubated with dsRNA in the absence of transfection reagent (32.5%). In contrast, transfection of KC cells using Lipofectamine 2000 gave <5% of transfected cells after plasmid DNA transfection, compared to 27% in GeneJammer and 11% in Fugene. No

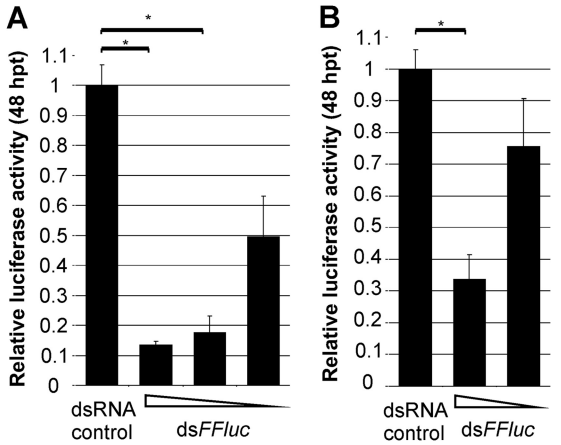

FIG 1 KC cells have a functional dsRNA-inducible RNAi pathway. KC cells were cotransfected with plasmids pAcIE1-Rluc and pIZ-Fluc. Following transfection, different concentrations of either eGFP-specific (ds-control) or *FFluc*-specific (ds*FFLuc*) dsRNA were either transfected (A) or added to the culture media 24 h posttransfection of plasmids (B). The relative luciferase expression (*FFluc/RLuc*) was determined at 48 h posttransfection (hpt). The mean with standard error is shown for two independent experiments performed in triplicate. *, $P < 0.05$, *t* test.

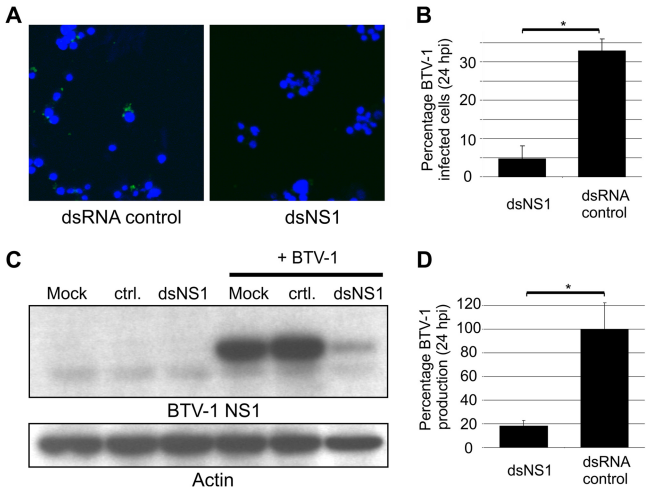

FIG 2 BTV-1 can be targeted by dsRNA in KC cells. (A) KC cells were transfected with either eGFP-specific control dsRNA or dsRNA targeting BTV NS1 (dsNS1), followed by BTV-1 infection at 24 h posttransfection. Twenty-four hours postinfection (hpi), cells were fixed and BTV-infected cells were visualized using a BTV NS1-specific primary antibody and an anti-rabbit secondary antibody conjugated to Alexa 488 (green signal). Cell nuclei were stained with DAPI. (B) Quantification of fluorescent cells. Infected and noninfected cells were counted, and the respective percentages from three independent experiments for each treatment were determined. (C) BTV-1 NS1 expression was determined by Western blot analysis in lysates of dsRNA-transfected and BTV-1-infected KC cells at 24 h postinfection using a BTV NS1-specific primary antibody. Western blot detection of actin was used as a loading control. (D) BTV-1 titers in cell culture supernatant, determined 24 h postinfection. Means from three independent experiments are shown; error bars represent standard errors. *, $P < 0.05$, *t* test.

green fluorescing cells were observed if plasmid DNA was added to cells in the absence of a transfection reagent.

Next, we determined the ability of dsRNA, either added to the medium or transfected with GeneJammer, to silence a firefly luciferase (*FFluc*) reporter gene. Cells were cotransfected with plasmids expressing *FFluc* as well as *Renilla* luciferase (*RLuc*) as an internal control expressed from baculovirus promoters (OpIE2 for *FFluc* in pIZ-Fluc and AcIE1 for *RLuc* in pAcIE1-Rluc). At 24 h postseeding, we induced an RNAi response by transfection (Fig. 1A) or addition (Fig. 1B) of different concentrations of either eGFP-specific control dsRNA (ds-control) or dsRNA targeting *FFluc*. We assessed luciferase activities 24 h after the addition of dsRNA. A concentration-dependent reduction of luciferase activity was found for cells treated with *FFluc*-specific dsRNA, regardless of whether dsRNA was transfected (Fig. 1A) or added to the cell culture medium (Fig. 1B). These results show that KC cells are able to induce a sequence-specific, dsRNA-dependent RNA silencing response. In addition, our data show that, similarly to what has been reported for Schneider-2 (S2) drosophila cells (56, 57), KC cells are able to take up dsRNA from culture medium.

The dsRNA-inducible RNAi response in midge cells displays antiviral activity. We next investigated the ability of this pathway to inhibit virus replication. As BTV is known to be transmitted to susceptible mammals via *Culicoides* species (27), we used this virus to investigate the dsRNA-induced antiviral RNAi response in KC cells. We first transfected the KC cells with dsRNA targeting BTV-1 NS1 or eGFP-specific control dsRNA. After 24 h, we infected the cells with BTV-1 at a multiplicity of infection (MOI) of 0.2. The success of silencing was assessed at 24 h postinfection by immunofluorescence (Fig. 2A) and Western blot detection (Fig. 2C) using an NS1-specific antibody. A reduction in NS1-positive cells was detected when dsRNA targeting NS1 was transfected and compared to control dsRNA. Approximately 35% of control dsRNA-transfected cells expressed BTV-1 NS1 as assessed by fluorescence microscopy, in contrast to 15% when dsRNA targeting NS1 was transfected (Fig. 2B). We confirmed these results by Western blotting, where levels of NS1 expression were greatly reduced in cells transfected with virus-specific dsRNA (Fig. 2C). BTV NS1 has been shown to be important for viral replication (58, 59), and consequently knockdown of NS1 expression will have a negative effect on virus production. We therefore measured BTV infectious viral particles released in the supernatant of KC cells incubated with dsRNA (targeting BTV-1 NS1 or control) at 24 h postinfection. As expected, a significant decrease in BTV-1 production was detected in cells incubated with dsRNA targeting NS1 compared to that of control infections (Fig. 2D). Similar results were obtained when we added (rather than transfected) dsRNA to the culture medium (data not shown), suggesting a capability for antivirally active dsRNA uptake similar to that observed for *Drosophila* cell lines (56, 57).

***Culicoides* cells can mount an RNAi response against the dsRNA bluetongue virus.** Having shown that RNAi can be induced in KC cells following dsRNA transfection, we investigated whether such a response could be induced following viral infection. Induction of an antiviral RNAi response is characterized by the production of small RNA molecules that map to the viral genome and/or antigenome (5, 6, 11, 12, 19, 20, 60). We isolated total RNA from KC cells 24 h postinfection with BTV-1, and we then sequenced small RNAs below 40 nt by Illumina Solexa sequencing and determined the sequences, frequencies, and BTV genome location. For most BTV-1 segments, the viRNA molecules produced in infected KC cells were predominantly 21 nt in length and mapped to both the coding and noncoding strand with similar frequencies (Fig. 3

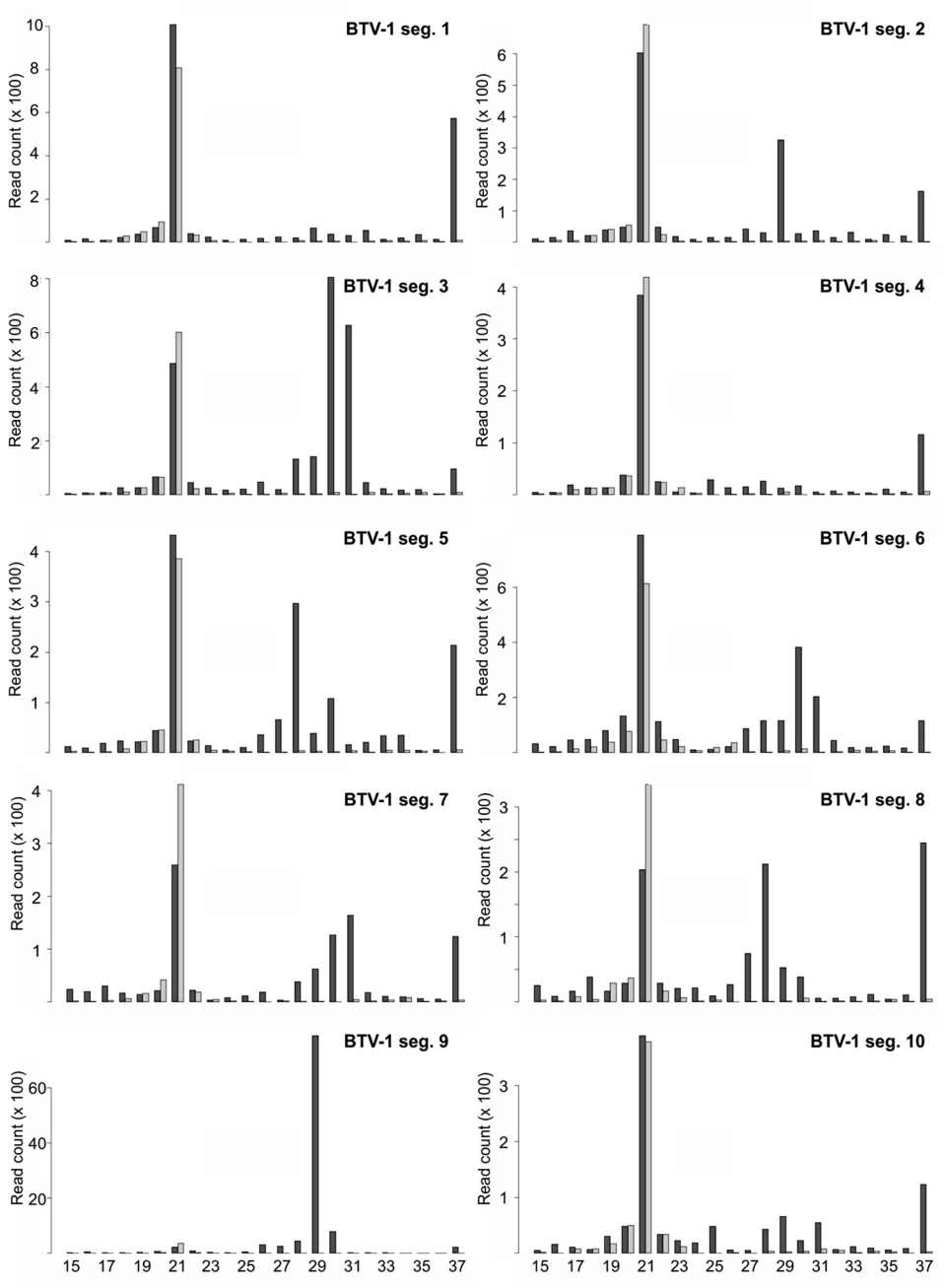

FIG 3 BTV-1 is targeted by the RNAi response in KC cells. Size distribution of small RNA molecules mapping to each segment (1 to 10) of BTV-1 in KC cells at 24 h postinfection. The *y* axis indicates the frequency of small RNAs; the *x* axis indicates the length in nucleotides. Dark grey indicates small RNAs mapping to the coding strand, and light grey indicates small RNAs mapping to the noncoding strand.

and 4). The 21-nt viRNAs were distributed along the coding and noncoding genome strand with variable frequency and a hot (high viRNA reads) and cold (low or no viRNA reads) spot distribution, similar to what has been described for mosquito-borne arboviruses (5, 6, 11, 12, 19, 20, 60). The frequency of the viRNAs was segment dependent. In addition to 21-nt viRNAs, other classes of small RNAs between 26 to 31 nt in length with a bias for the BTV-1 coding strand were also identified. The frequency of these longer RNAs differed per segment from few (segment 1) to the majority of virus-specific small RNAs (segment 9) (Fig. 3 and 4). To determine if these results were specifically induced by BTV, we investigated the produced small RNAs against BTV-1 in the nonvector cell line Aag2 (derived from *Aedes aegypti*). We established by fluorescence microscopy that BTV-1 was able to infect Aag2 cells at levels comparable to KC cells (Fig. 5). RNA was isolated 48 h postinfection, and small RNAs were sequenced on the Illumina sequencing platform as described before. The viRNA production pattern,

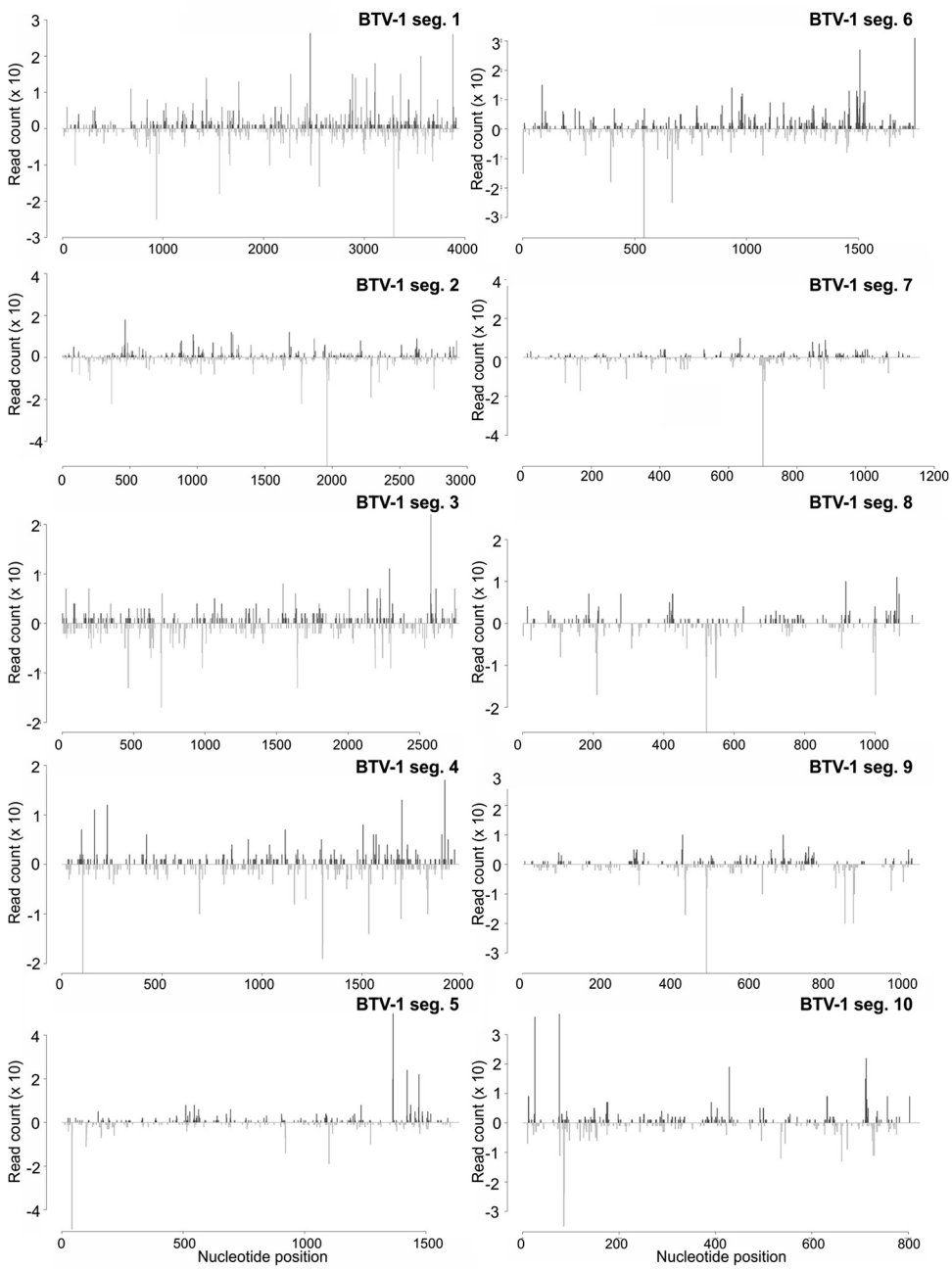

FIG 4 Distribution of 21-nt viRNAs of BTV-1 in KC cells. Frequency distribution of 21-nt viRNAs to each segment (1 to 10) of BTV-1. The *y* axis shows the frequency of the 21-nt viRNAs mapping to the corresponding nucleotide position on the *x* axis. Positive numbers and peaks represent the frequency of viRNAs mapping to the coding strand (in 5′→3′ orientation). Negative numbers and peaks represent those viRNAs mapping to the noncoding strand (in 3′→5′ orientation).

including the larger class of virus-specific small RNAs, was largely conserved in Aag2 cells infected with BTV-1 (Fig. 6 and 7). Importantly, pattern, location, and frequencies of viRNA production were conserved mainly between independent experiments (data not shown). Together, these data show that the antiviral RNAi responses following infection by BTV of *C. sonorensis*-derived KC cells and *Aedes aegypti*-derived Aag2 cells are broadly comparable and are predominantly characterized by the production of 21-nt viRNAs for most of the segments.

***Culicoides* cells can mount an RNAi response against the negative-strand RNA Schmallenberg virus.** SBV is a recently emerged pathogen belonging to the *Orthobunyavirus* genus of the *Bunyaviridae* family and is thought to be most probably midge-borne (4). We infected KC cells with SBV, and RNA was isolated 24 h postinfection to determine if a similar production pattern of viRNA production is observed in KC cells infected with an arbovirus belonging to a different virus family from the *Reoviridae*. Infection experiments indicated that SBV infects and replicates

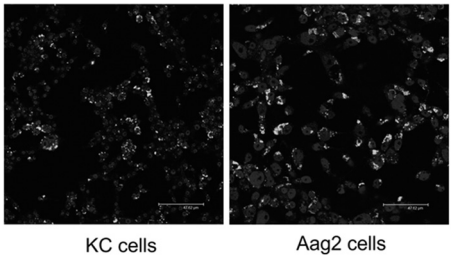

FIG 5 BTV-1 infects nonvector mosquito Aag2 cells. BTV-infected KC and Aag2 cells were fixed at 48 h postinfection, and expression of NS1 protein was monitored by immunofluorescence (bright signal). Cell nuclei were stained with DAPI.

with low frequency in KC cells even after high multiplicity of infection (Fig. 8 A and B). Similar experiments performed on Aag2 cells showed enhanced infection and replication of SBV (Fig. 8A and B). Therefore, SBV-infected Aag2 cells were used in order to investigate if the results obtained for BTV-1-infected KC and Aag2 cells could be broadened to another arbovirus. Sequences, frequencies, and SBV genome locations of small RNAs below 40 nt were determined as described above. As shown in Fig. 8C, 21-nt viRNAs are the predominant species of viRNA in KC cells and for most of the segments in Aag2 cells. Again, a distribution along the L, M, or S genome and antigenome with hot and cold spots was observed (Fig. 9A). In both KC and Aag2 cells, most viRNA reads are generated by the S segment, followed by the M and L segments (Fig. 8C). In addition to 21-nt viRNAs, small RNAs in the size range of 24 to 30 nt with a bias for the positive antigenome strand were detected in Aag2-infected cells and matched with all three viral segments, although at different frequencies. In the case of the S segment, these larger small RNAs represented the majority of small RNAs (Fig. 8C). As described above, similar class sizes of small RNAs were also detected in BTV-1-infected KC and Aag2 cells. This size range of small RNAs normally represents the group of PIWI-interacting RNA (piRNA) molecules, known to be important in suppressing transposons in germ line cells in various organisms, including drosophila, zebrafish, and mice (61–65). Primary piRNAs are normally antisense to the genomic regions (mostly transposons) and target transposon-derived single-stranded sense RNA. Upon cleavage, secondary piRNAs are produced that are mostly sense and used to find complementary antisense RNA, resulting again in primary-type piRNAs. Recently, virus-specific piRNA-like molecules have been reported for several arboviruses, including CHIKV, Sindbis virus (SINV), and LACV in aedine mosquitoes or their derived cell lines (6, 18, 19, 44). Due to the so-called "ping-pong" mechanism of piRNA production, piRNAs have specific features. The primary piRNAs are in antisense orientation and have a bias for uridine at position 1. In contrast, secondary piRNAs are in the sense orientation and have an adenine at position 10. In addition, complementary piRNAs and viral piRNA-like molecules of LACV and SINV are often separated at the 5′ end by 10 nucleotides (44, 66). Most of the SBV-specific small RNAs of 24 to 30 nt produced in Aag2 cells have piRNA-specific features (sense [antigenome] with A_{10} and antisense [genome] with U_1 [Fig. 10A] and separation of complementary RNAs at the 5′ end by 10 nucleotides [Fig. 10B]), in particular those produced from the M and S segments. They are distributed along the segments and do not map to a specific region of the segments (Fig. 9B). In contrast, the BTV-1-specific 24- to 31-nt small RNAs produced in KC and Aag2 cells do not show any specific sequence logo (data not shown). Pattern, location, and frequencies of viRNA production were conserved mainly between independent repetitions (data not shown).

Taken together, our data show that midge-derived KC cells mount an antiviral RNAi response following infection with arboviruses with different genome structures. Regardless of the virus infecting these cells, viRNAs of 21 nt in length were found to be the dominant class of virus-specific small RNA. Thus, the *Culicoides* antiviral RNAi response resembles mainly similar pathways found in mosquitoes. Larger small RNAs with features of piRNA-like molecules were found for SBV-infected Aag2 cells but not KC cells. Although similar RNA molecules were detected in BTV-infected KC and Aag2 cells, these RNAs do not possess piRNA-specific features.

DISCUSSION

RNAi (and in particular the exogenous RNAi pathway) has been shown to be a major antiviral response against arboviruses in mosquitoes and an important process regulating this virus/host interaction (13, 14). *Culicoides* midges are one of the major invertebrate vectors of several arboviruses of humans and livestock. In this study, we have shown that *C. sonorensis*-derived KC cells mount an antiviral RNAi response.

Drosophila cells have been reported to take up dsRNA from culture medium, a phenomenon not observed for any of the mosquito-derived cell lines (56, 57). Interestingly, we show that also KC cells are able to take up dsRNA molecules directly from the culture medium, although the precise pathway for dsRNA uptake is not known. Several genes have been linked with the dsRNA uptake in *D. melanogaster*, suggesting receptor-based endocytosis (56, 57, 67). Due to the lack of genomic information on *Culicoides*, it is currently difficult to draw any comparisons.

dsRNA and siRNA molecules have been used to target and silence a variety of viruses in numerous organisms. Even mammalian cells, believed to not naturally mount a siRNA-based antiviral RNAi response upon viral infection, can induce an siRNA-based antiviral RNA silencing response after transfection of siRNA molecules (68). Therefore, successful inhibition of virus production following transfection of dsRNA targeting viral sequences cannot be used solely as an indication that such an antiviral RNA silencing response occurs during natural infection. A key feature of antiviral RNAi in mosquitoes (as in other insects) is the production of 21-nt viRNA molecules (13, 14). Deep sequencing of KC cells infected with two different midge-borne arboviruses (BTV and SBV) shows that the majority of viRNAs for most BTV genomic segments (8 out of 10) and all SBV segments are 21 nt in length. viRNAs in mosquito cells (and other insects) are produced by Dcr-2 cleavage of dsRNA molecules that could derive either from replicative intermediates or secondary structures within the viral transcripts (14) but also the viral genome itself in case of dsRNA viruses such as BTV. From the available viRNA profiles, replication intermediates are generally the favored Dcr-2 substrate candidates. This is supported by the scattering of viRNAs across the whole genome/antigenome, the presence of hot and cold spots of viRNA production (5, 6, 11, 12, 19, 20, 69), and no real preference of a longer stretch of only the genome or antigenome as the producer of 21-nt RNAs, which would be expected if secondary RNA structures are the favored Dcr-2 substrate. Recently, it has been

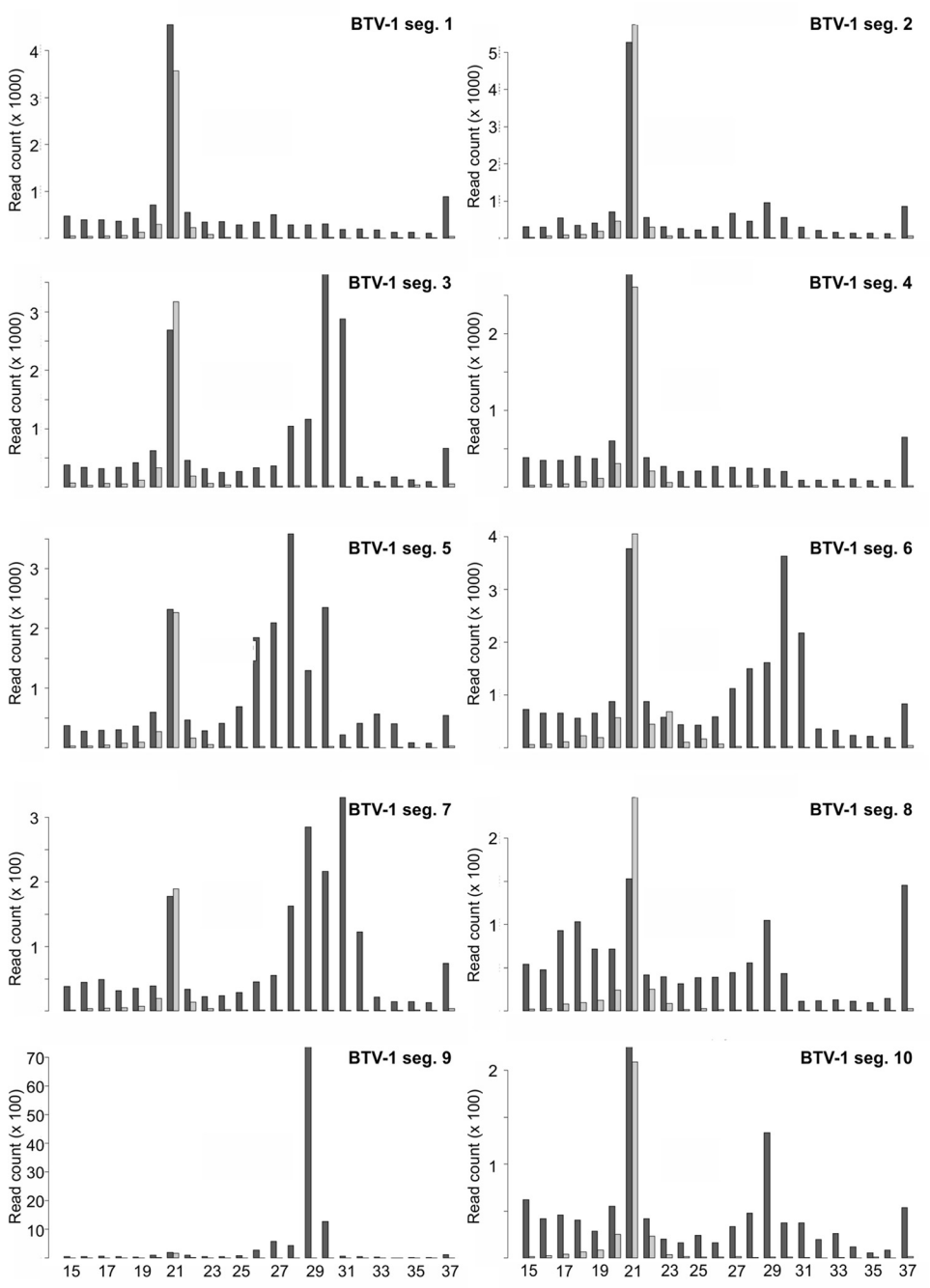

FIG 6 BTV-1 infection is targeted by the RNAi response in Aag2 cells. Size distribution of small RNA molecules mapping to each segment (1 to 10) of BTV-1 in Aag2 cells at 48 h postinfection. The *y* axis indicates the frequency of small RNAs; the *x* axis indicates the length in nucleotides. Dark grey indicates small RNAs mapping to the coding strand, and light grey indicates small RNAs mapping to the noncoding strand.

shown that certain RNAs can be over- or underrepresented in small RNA libraries, due to low sequencing depth and cloning bias (70, 71). Some of the observed hot and cold spots could be the result of such a cloning bias; however, the presence of small RNAs mapping to the noncoding strand of BTV with a similar frequency as to the coding strand strongly supports the dsRNA genome as the RNAi inducer molecule. Before this study, it was not immediately apparent whether an RNAi response of 21-nt viRNAs would be induced following infection by viruses with a dsRNA genome like the orbivirus BTV and if the inducer molecules would be the dsRNA genome or secondary structures in the viral transcripts. Considering that synthesis of the negative-sense RNA during the viral replication cycle is believed to occur only within the newly assembled viral particles, the secondary structures of the viral transcripts would be the favored RNAi inducer molecule; this strategy helps dsRNA viruses also to shield them from host pro-

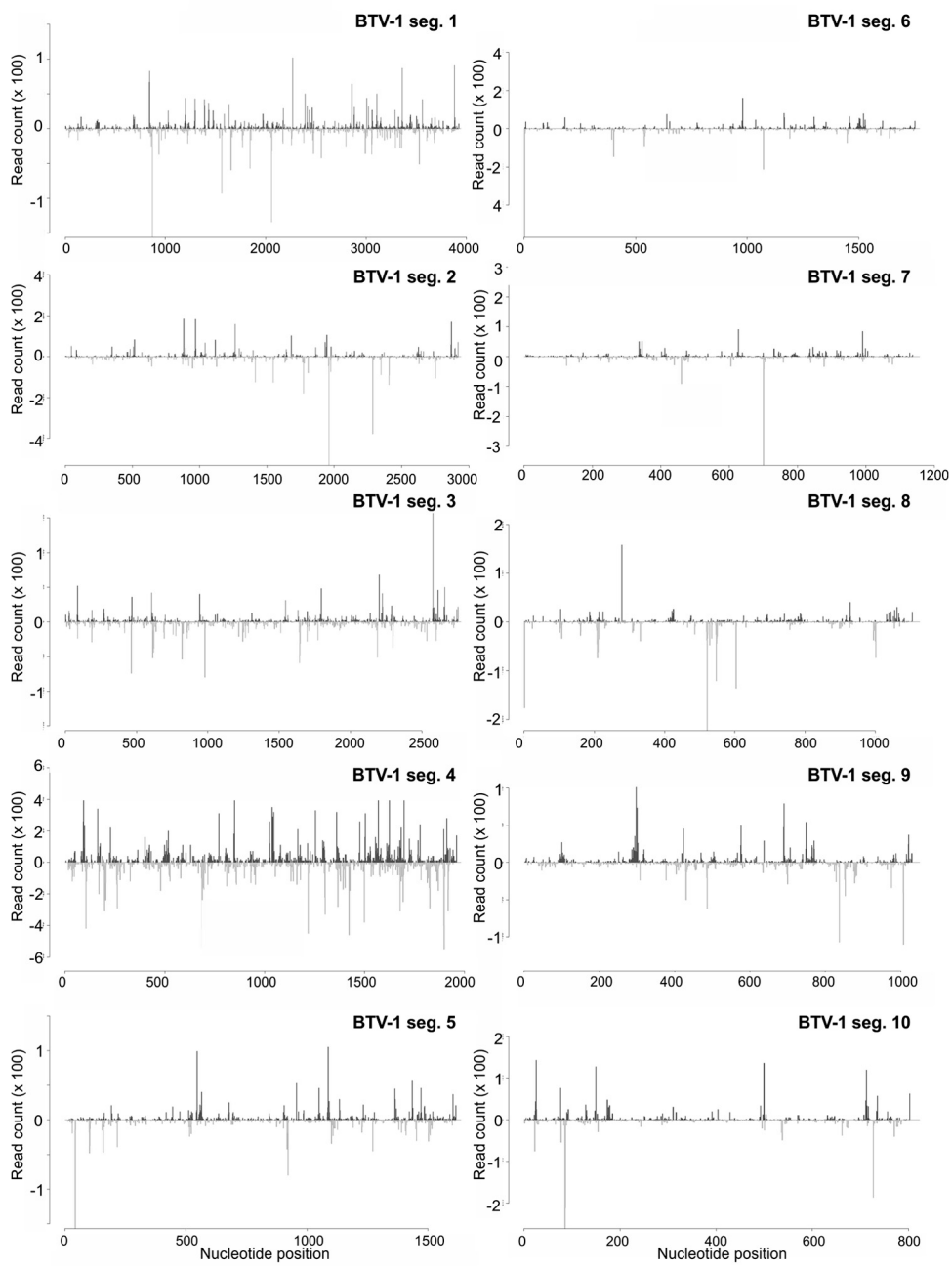

FIG 7 Distribution of 21-nt viRNAs of BTV-1 in Aag2 cells. Frequency distribution of 21-nt viRNAs in infected Aag2 cells to each segment (1 to 10) of BTV-1. The *y* axis shows the frequency of the 21-nt viRNAs mapping to the corresponding nucleotide position on the *x* axis. Positive numbers and peaks represent the frequency of viRNAs mapping to the coding strand (in 5′→3′ orientation). Negative numbers and peaks represent those viRNAs mapping to the noncoding strand (in 3′→5′ orientation).

teins such as RNA sensors that activate the antiviral interferon pathway in mammals (72–76). Thus, viRNAs of dsRNA viruses could have been solely derived from the secondary structures of the coding RNA strand (which functions as mRNA), or these viruses could escape antiviral RNAi altogether (72, 75, 76). The features of BTV viRNAs detected in our study (scattering across the whole genome in sense and antisense patterns) indicate that at least a small amount of dsRNA viral genome is accessible to the RNAi machinery and probably not protected by the double-layered viral membrane particles, as recently shown for the induction of interferon (IFN) in mammalian cells by BTV dsRNA (77). These results are in line with the detection of viRNAs derived from dsRNA viruses (the birnavirus drosophila X virus [DXV] as well as drosophila totivirus) (78) during persistent infection in a drosophila cell line and the increase in susceptibility for DXV in RNAi-deficient drosophila (79). The resulting viRNAs are expected to be able to target the BTV transcripts present in the cytoplasm, resulting in less viral protein production and subse-

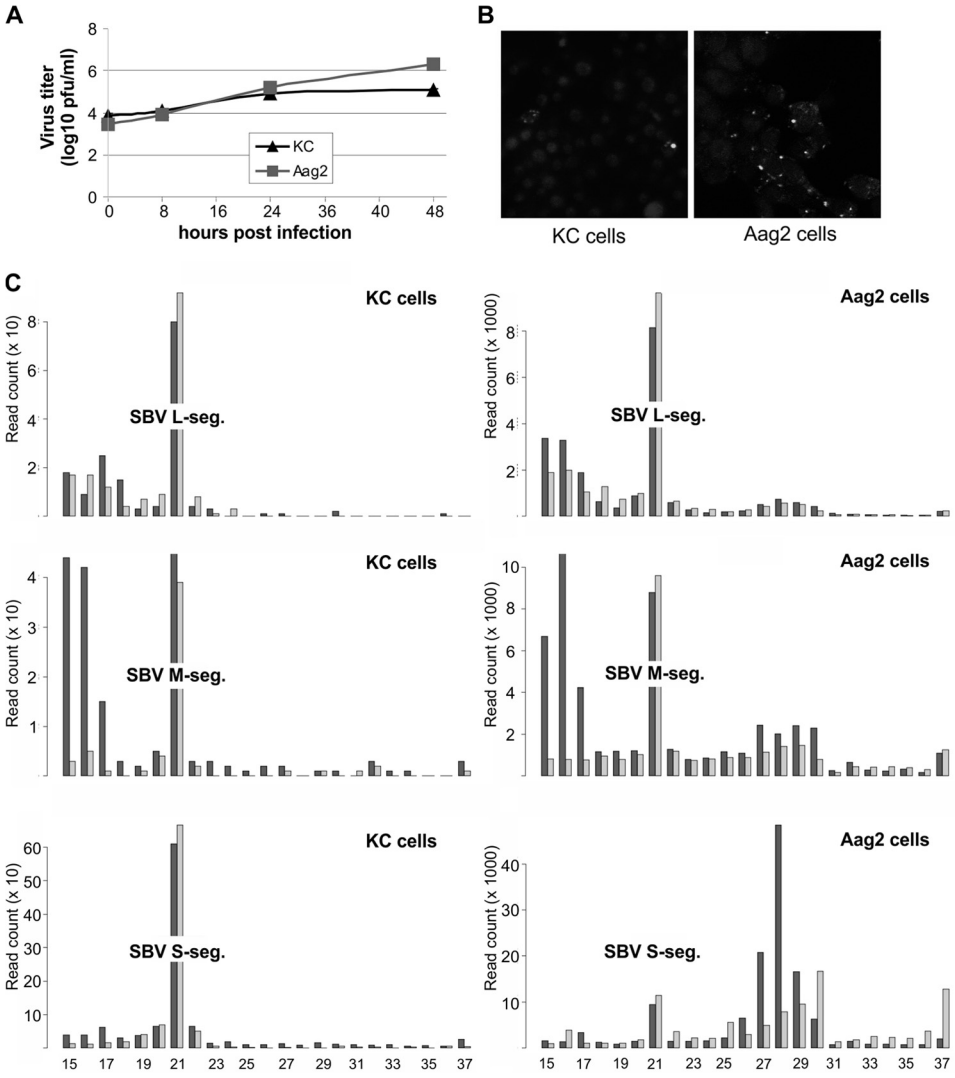

FIG 8 SBV infection and targeting by the RNAi response in KC and Aag2 cells. (A) Virus production in supernatant of KC (triangle) and Aag2 (square) cells infected with SBV at a MOI of 10 was determined at various time points postinfection (0, 8, 24, and 48 h) by plaque assay. A representative of two independent experiments performed in triplicate is shown; standard errors are indicated. (B) SBV-infected KC and Aag2 cells were fixed at 48 h postinfection, and SBV-infected cells were visualized by using an SBV N-specific primary antibody (bright signal). Cell nuclei were stained with DAPI. (C) Size distribution of small RNA molecules mapping to L, M, and S of SBV in KC cells at 24 h postinfection or in Aag2 cells at 48 h postinfection. The *y* axis indicates the frequency of small RNAs; the *x* axis indicates the length in nucleotides. Dark grey indicates small RNAs mapping to the coding strand, and light grey indicates small RNAs mapping to the noncoding strand.

quently reduced virus titers, as shown in this study. In addition to the 21-nt viRNAs, larger classes of BTV-specific small RNAs of 26 to 31 nt in length were detected. This resembles the size distribution of piRNAs, a class of Dicer-independent small RNAs found in vertebrate and invertebrates thought mainly to be important for genome stability in germ line cells by targeting transposons (61–65). Recently, virus-specific piRNA-like small RNAs were found in arbovirus-infected aedine mosquitoes and derived cell lines. These piRNA-like molecules were found to map mainly to the coding strand of the viral genome of positive-strand RNA arboviruses but also to the antigenome of LACV (18, 19, 44). Due to their production pathway (the so-called ping-pong amplification mechanism), piRNAs and viral piRNA-like molecules have a specific sequence logo (61–65). BTV-specific small RNAs of 26 to 31 nt in length do not show a piRNA-like sequence logo (not shown). It is not known how these larger BTV-specific RNA molecules are produced, or their function, or if they are related to RNAi processes at all. Their production is virus specific and not cell type specific, as we also detected them in the Aag2 mosquito cell line.

In the case of SBV, 21-nt viRNAs could be detected in both KC and Aag2 cells for all three segments in an asymmetric distribution, suggesting again a dsRNA replication intermediate as the inducer of the RNAi response. This is in line with results observed in drosophila cells infected with LACV (6). Compared to infection with positive-strand RNA viruses or dsRNA viruses, no dsRNA could be detected for negative-strand RNA viruses, at least in infected vertebrate cells (80). Our results indicate that dsRNA replicative intermediates are present in orthobunyavirus-infected cells, possibly at low levels but levels still sufficient to induce an antiviral RNAi response. Longer small RNA molecules containing

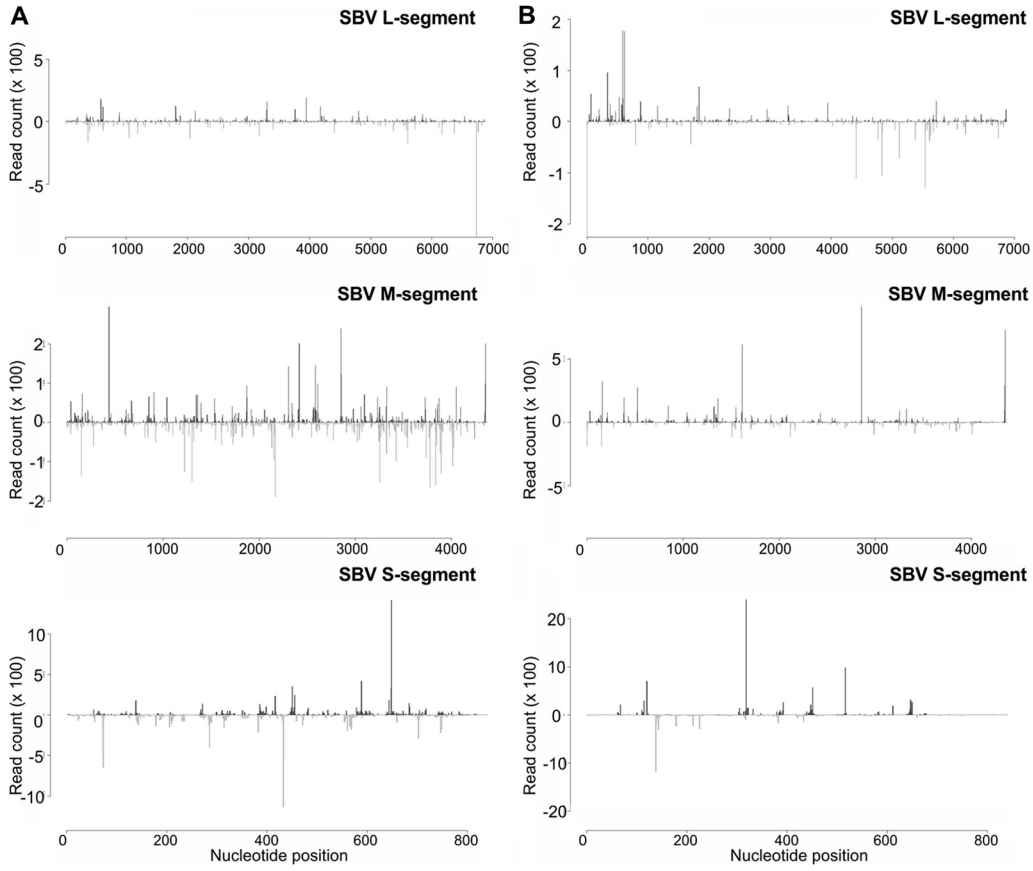

FIG 9 Distribution of SBV-derived small RNAs in Aag2 cells. Frequency distribution of 21-nt viRNAs (A) or 24- to 29-nt small RNAs (B) to L, M, and S of SBV in Aag2 cells at 48 h postinfection. The *y* axis shows the frequency of the 21-nt viRNAs mapping to the corresponding nucleotide position on the *x* axis. Positive numbers and peaks represent the frequency of viRNAs mapping to the coding strand (in 5′→3′ orientation), and negative numbers/peaks indicate those viRNAs mapping to the noncoding strand (in 3′→5′ orientation).

piRNA features were also detected in SBV-infected Aag2 cells, as recently described for other bunyaviruses (LACV, Rift Valley fever virus [RVFV]) in RNAi-deficient *Aedes albopictus*-derived C6/36 cells and other arboviruses (such as CHIKV, SINV, DENV, and RVFV) in aedine mosquitoes and/or their derived cell lines (18, 19, 44, 81). As no piRNA-like molecules could be detected in any of the BTV-1-infected cell lines, production of piRNA-like molecules may be specific to single-stranded RNA viruses, though more research is needed to answer this question. It is not known if these piRNA-like molecules have an antiviral function and how they are induced. Neither BTV nor SBV produced piRNA-like molecules in KC cells. This could be due to either the lack of a piRNA pathway in *Culicoides* species or, alternatively, a deficiency of the KC cell line.

The detection of BTV- or SBV-specific 21-nt viRNAs indicates the ability of KC cells to induce an antiviral RNAi response. In the absence of any genomic information on *Culicoides*, we can only speculate that orthologs of exogenous RNAi pathway proteins such as Dcr-2 or Ago-2 are present in the midge genome. However, the presence of 21-nt viRNAs is a strong indicator for the presence of an exogenous RNAi pathway that is comparable to that of mosquitoes (14). This raises a number of questions: most importantly, how is BTV or SBV able to successfully replicate and infect its midge vectors? Plant and true insect viruses have been reported to encode proteins able to interfere with the antiviral RNA silencing response by expressing RNA silencing suppressor proteins (RSS) (23, 82). Until now, no RSS protein has been identified in arboviruses, suggesting other strategies for inhibition or evasion of the RNAi response. Previous data obtained from SFV infection of mosquito cells suggests a decoy mechanism: viRNAs that are produced at high concentrations are not able to target the virus efficiently in contrast to viRNAs produced at low concentrations that result in efficient silencing of the virus. This strategy results in successful replication at least for some time postinfection, even though viRNAs are produced (12). The mosquito-borne flaviviruses WNV and DENV have been recently shown to express a subgenomic flavivirus RNA able to interfere with the RNAi response in mosquito cells, thereby ensuring efficient viral replication (83). However, all mosquito-borne arboviruses investigated thus far are efficiently targeted by the RNA silencing response (5, 6, 11, 12, 19, 20, 60). Further research is needed to determine if and whether SBV or BTV do have any (even if weak) RNAi evasion/inhibition strategies.

Taken together, our findings define for the first time the presence of an RNAi response in *Culicoides* cells which is able to target midge-borne arboviruses and resembles at least in part the exogenous antiviral RNAi pathway of mosquitoes. More work will be

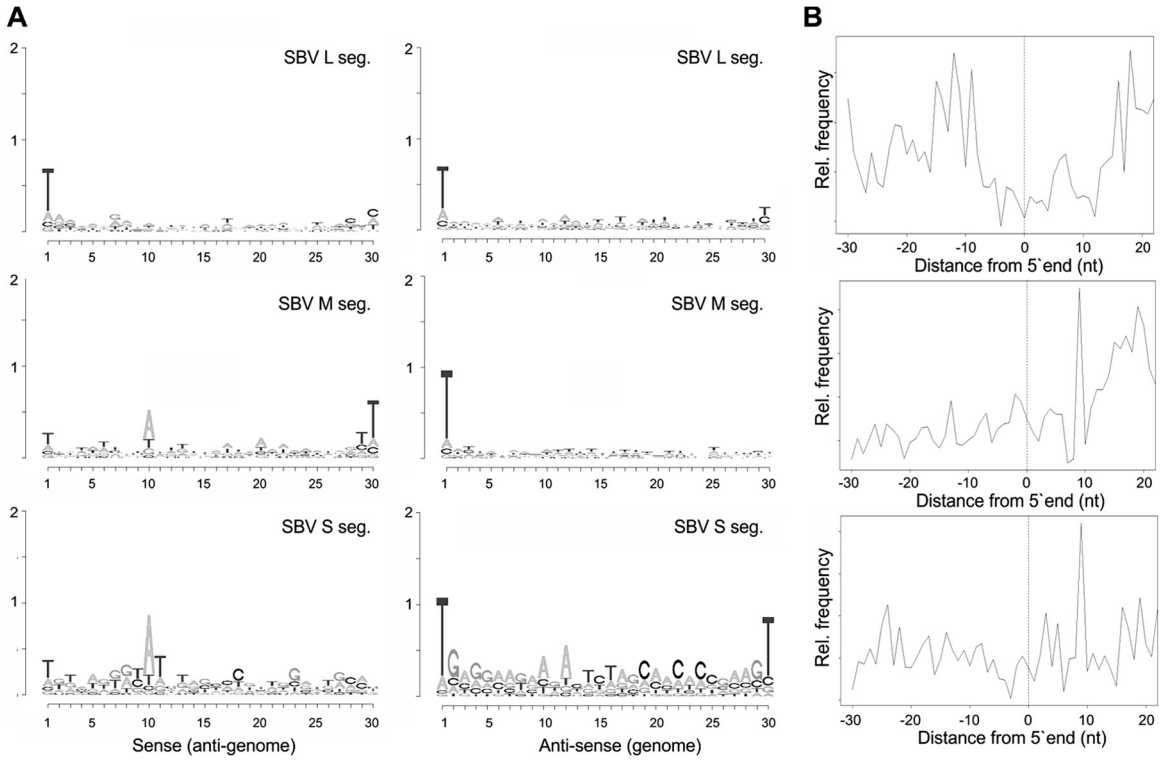

FIG 10 SBV-specific piRNA-like molecules produced by viral infection in Aag2 cells. (A) Relative nucleotide frequencies and conservations per position of 24- to 30-nt small RNAs mapping to the antigenome (left) and genome (right) of SBV in Aag2 cells are indicated for L, M, and S. The overall height of the nucleotide represents the sequence conservation. Note: the sequence is represented as DNA. (B) Frequency mapping of the distance of complementary 24- to 30-nt small RNAs mapping to SBV segments (top to bottom: L, M, and S) in Aag2 cells.

required to determine the exact mechanisms and proteins involved in the RNAi pathway in midges, but this study allows further investigations into these processes.

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